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In vitro sterilization of Lilium LA Hybrids "Indian Summerset" and "Nashville" as influenced by different sterilant combinations

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Abstract
The present study was conducted in order to develop an efficient protocol for sterilization of bulb scale and leaf segment explants of LA Hybrid cultivars of Lilium, "Indian Summerset and "Nashville". Surface sterilization of the scales of flowering bulbs and segments of young leaves of both the cultivars was carried out with eight different combinations of surface sterilants at varying concentrations and time duration of the exposure. The surface sterilants used under the study were Carbendazim, Mercuric chloride and Ethyl alcohol Maximum asepsis in bulb scale explants was achieved with the application of Carbendazim 0.02% for 30 minutes followed by Mercuric chloride 0.1% for 10 minutes and then by ethyl alcohol 70% for 10 seconds. In case of leaf segments, maximum asepsis was recorded with the application of similar treatment combinations except in case of Mercuric chloride exposure which was reduced to 5 minutes. Highest survival was recorded with application of Carbendezim 0.02% for 20 minutes, followed by Mercuric chloride 0.1% for 10 minutes and then by ethyl alcohol 70% for 10 seconds in case of bulb scale explants while as in case of leaf scale explants highest survival was recorded with same treatment combinations except for exposure of mercuric chloride treatment for 2 minutes.

Keywords: Asepsis, carbendazim, ethanol, *in-vitro*, LA hybrid, lilium, mercuric chloride, survival, tissue culture

Introduction
Lilium is one of the world's most popular cut flower and its commercial importance stems from its bold, beautiful, and fascinating flower form, long vase life, and ability to rehydrate after long transportation. Bulbs are commercially produced for use in the cut-flower and pot plant sector. They are also used as a patio plant and in herbaceous borders, woodlands, and shrub plantings. Considering the commercial importance and demand for novel varities and disease free flowers, tissue culture techniques come into play for disease free mass scale production. Lilium tissue culture began in the late 1950s (Robb, 1957) [18] and has been used successfully for rapid propagation in a variety of Lilium species and cultivars, including *L. Longiflorum*, Oriental and Asiatic and Longiflorum- Asiatic (LA) Hybrid lilies (Lian *et al*., 2002; Bacchetta *et al*., 2003) [11, 2]. *In vitro* scale culture is one of the most effective and prolific vegetative propagation methods for lilies (Bahr and Compton, 2004) [3]. Micropropagation in lilium allows for large-scale bulblet production all year long under controlled environmental conditions. Using tissue culture, one large bulb can yield approximately one million small bulblets in two years (Langens-Gerrits, 2003) [9].
Selecting the appropriate explant is critical if the desired outcome of any tissue culture process is to be accomplished with limited delays. Additionally proper sterilisation of the explants is a necessary step in the development of a successful protocol for *in vitro* propagation. Plants contaminated with pathogens have a lower multiplication, survival, and regeneration rate, as well as a deteriorated quality of plant genetic resources (Wang and Valkonen, 2009) [25]. One of the most serious issues in the micro-propagation of any plant species is the fungal and bacterial contamination. Microbial and fungal contamination can be caused by a variety of factors, including infected plant materials, faulty tissue culture techniques, and poor laboratory conditions (Shen *et al*., 2010; Tomas *et al*., 2011; Fang *et al*., 2012) [20, 5]. Explant contamination is caused by the factors including the source of the explants and the growing environmental conditions (Tyagi *et al*., 2011, Chen *et al*., 2011) [24, 4].

In vitro cultures the pathogens compete with plants for nutrients readily available in the media (Omamor *et al*., 2007) [15] and the growing conditions are also conducive for the completion of pathogen lifecycle. The presence of these pathogens increases plant mortality causes growth variation (reduced shoot proliferation and rooting), tissue necrosis, and even plant death. The contaminant in culture media may manifest itself immediately or latently, remaining dormant for an extended period of time (Leifert and Cassells, 2001) [10]. Epiphytic bacteria are found on plant surfaces and can be removed using chemical disinfectants (Hirano and Upper, 1990) [8]. Endophytic microbes, on the other hand, colonise living internal tissues of plants without causing immediate damage (Sette *et al*., 2006) [19] and are not easily eliminated by simple surface sterilisation methods. As a result, existing contaminants are typically controlled with antibiotics or fungicides under *in vitro* conditions (Niedz, 1998) [14].

Thus to establish any *in vitro* micropropagation protocol, a proper sanitation in the culture laboratories during the inoculation sessions as well as the standardization of the sterilant treatments for different explant types need to be optimized. During the current investigation, various sterilants were used to improve the culture asepsis in Lilium in order to standardize the sterilant treatment combination and its exposure durations for two L.A hybrid cultivars of Lilium thus generating a platform for further tissue culture experiments.

Materials and Methods

The present study was carried out in the Plant Tissue Culture Laboratory of the Division of Floriculture and Landscape Architecture, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, during the year 2020. Explant sources for the experiment included mature, flowering-sized bulbs and young leaves from two LA hybrids, "Indian Summerset" and "Nashville."

Culture media (Murashige and Skoog media (Murashige and Skoog, 1974)) [13] in test tubes were sterilised by autoclaving for 20 minutes in a vertical autoclave at 121°C and 1.05 kg cm ^{2} (15 psi), respectively. All aseptic manipulations, such as surface sterilisation, explant preparation and inoculation, were performed in the laboratory using a laminar air flow cabinet. Healthy outer scales and young leaves from the said cultivars were used for the experiment. The bulb scales and leaves were placed in a beaker and washed with running tap water in the laboratory to remove any adhering dirt and contamination before the explants were isolated. Before further processing, the explants were placed in clean flasks containing distilled water and shaken vigorously for 30 minutes in a Tween-20 surfactant followed by the treatment with different concentrations of Carbendazim for different exposure durations. The surfactant and fungicide were rinsed away with running tap water, followed by a final wash with single distilled water. Following initial cleaning, the explants were transferred to a laminar air flow hood for further surface sterilant treatments that included mercuric chloride and ethanol at different concentrations and exposure durations. The bulb scales and the leaves were cut in small uniform sized discs and put on the media to evaluate the effects of the treatment combinations and their durations on asepsis and survival of the explants. The experiment was conducted in Completely Randomised Design (CRD) (Gomez and Gomez, 1984) with three replications and the data generated was statistically analysed using two factorial analysis with cultivar as one factor and sterilant treatments as the second. To meet model assumptions for analysis of variance, percentage data was transformed using the angular or square root transformations proposed by Steel and Torrie (1980) [21]. The data was recorded after 2 weeks of the culture for evaluation of asepsis per cent and after 4 weeks for survival per cent.

Results and Discussions

The effect of sterilant treatment combinations, cultivars and their interaction on culture asepsis per cent was observed and perusal of data revealed the significant difference among sterilant treatment combinations and cultivars in both the explants (bulb scale and leaf). Interaction between the two factors was also observed to be significant. Significantly highest culture asepsis in both the cultivars was recorded in treatment combination SB_8 (Application of Carbendazim 0.02% for 30 minutes followed by Mercuric chloride 0.1% for 10 minutes and then ethyl alcohol 70% for 10 seconds) in bulb scale explants (Table 1) and in case of leaf segment explants, significantly higher culture asepsis in both the cultivars was recorded in treatment combination SL_8 (Application of Carbendazim 0.02% for 30 minutes followed by Mercuric chloride 0.1% for 5 minutes and later treated with ethyl alcohol 70% for 10 seconds) while remaining at par with SL_7 (Carbendazim 0.02% for 20 minutes followed by Mercuric chloride 0.1% for 5 minutes and later treated with ethyl alcohol 70% for 10 seconds) (Table 2).

The evaluation of survival per cent of both the explants under the influence of sterilant treatment combinations, cultivar and their interaction was done and it was observed that the sterilant treatment combination had significant effects on the explant survival percentage in both the cultivars and the interaction between the two factors was also observed to be significant. However the effect of cultivar alone on survival of leaf segment explant was recorded to be non-significant. Maximum survival in bulb scale explants of both the cultivars was observed in SB_7 (Application of Carbendazim 0.02% for 20 minutes followed by Mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 10 seconds) (Table 1) and in case of leaf segment explants, highest survival was observed to be in SL_5 (Application of Carbendazim 0.02% for 20 minutes followed by Mercuric chloride 0.1% for 2 minutes and ethyl alcohol 70% for 10 seconds) in both the cultivas (Table 2).

During the present investigation, various sterilants alone or in combinations in varying exposure durations were used and evaluated for asepsis and survival of the bulb scale and leaf segment explants after 2 and 4 weeks respectively. Endophytic pathogens act as latent infections in the later stages of the cultures and cause contamination to the cultures (Farooq *et al*., 2021) [6]. Combined application of two or more sterilants have been found to be effective for sterilising underground buds/explants, as compared to single-chemical sterilisation (Rather, 2014) [17]. In a study conducted by Rafiq *et al*., (2021) [16], it was reported that the lilium bulb scales when treated with fungicide followed by ethanol wash produced significantly aseptic cultures. Sindhu *et al*. 2015 also used Carbendazim and Mercuric chloride treatments for the surface sterilization of the lilium bulb scales for establishing the micropropagation protocol for the cultivar “Pollyana”. In an Asiatic Hybrid cultivar “Red Alert”, maximum asepsis was reported when 0.1% Mercuric chloride

was used as a sterilant (Taha *et al*., 2018) [23]. Carbendazim and 75% ethanol had proved to be significantly effective treatments for asepsis of LA Hybrid "Eyeliner" (Liu *et al*., 2012) [12]. The combined treatments have resulted in optimum sterilization and survival in explants of *Lilium orientalis* and *Lilium longiflorum* cv. "White Fox" (Aslam *et al*., 2013) [1].

It was also observed in the present study that the sterilant treatment combination that resulted in maximum asepsis did not perform similarly for survival percentage. This may be attributed to the toxicity effect of the chemical sterilants at higher concentrations on the sensitive plant tissues in *in vitro* cultures (Rather *et al*., 2014) [17]. Thus the concentrations and combinations of the sterilants treatments need to be optimised for proper growth and development of disease and virus free propagation of the plants *in vitro*.

Table 1: Influence of different sterilant treatment combinations and cultivars on asceptic culture and survival of bulb scale explants of Lilium

Sterilant Treatments Combinations		Cultivars			
		Indian Summerset (C_1)	Nashville (C_2)	Indian Summerset (C_1)	Nashville (C_2)
		Asepsis %		Survival %	
SB_1	Mercuric chloride 0.1% for 5 minutes	12.5 (20.69)*	12.5 (20.69)*	39.58 (6.36)**	39.58 (6.36)**
SB_2	Mercuric chloride 0.1% for 10 minutes	18.74 (25.62)*	20.83 (27.14)*	50.00 (7.14)**	52.08 (7.28)**
SB_3	Mercuric chloride 0.1% for 5 minutes followed by ethyl alcohol 70% for 10 seconds	27.08 (31.33)*	29.16 (32.63)*	66.66 (8.22)**	64.58 (8.09)**
SB_4	Mercuric chloride 0.1% for 10 minutes followed by ethyl alcohol 70% for 10 seconds	31.24 (33.96)*	39.58 (38.96)*	56.24 (7.56)**	60.41 (7.83)**
SB_5	Carbendezim 0.02% for 20 minutes + S_3	49.99 (44.97)*	47.91 (43.78)*	79.16 (8.95)**	85.41 (9.29)**
SB_6	Carbendezim 0.02% for 30 minutes + S_3	81.24 (64.33)*	79.16 (62.81)*	68.41 (8.33)**	75.00 (8.71)**
SB_7	Carbendezim 0.02% for 20 minutes + S_4	56.24 (48.57)*	56.24 (48.57)*	91.66 (9.62)**	91.66 (9.62)**
SB_8	Carbendezim 0.02% for 30 minutes + S_4	85.41 (67.55)*	89.58 (71.19)*	72.91 (8.59)**	72.91 (8.59)**
	$C.D_{(P\leq0.05)}$ Cultivar (C)	0.85		0.064	
	Sterilant (S)	1.70		0.12	
	CX S	2.41		0.18	

*Figures in the parentheses are the statistically arcsin transformed values of percentage data
**Figures in the parentheses are the statistically square root transformed values of percentage data

Table 2: Influence of different sterilant treatment combinations and cultivars on aseptic culture and survival of leaf segment explants of Lilium

Sterilant Treatments Combinations		Cultivars			
		Indian Summerset (C_1)	Nashville (C_2)	Indian Summerset (C_1)	Nashville (C_2)
		Asepsis %		Survival %	
SL_1	Mercuric chloride 0.1% for 2 minutes	39.58 (6.36)**	39.58 (6.36)**	37.50 (37.74)*	37.50 (37.74)*
SL_2	Mercuric chloride 0.1% for 5 minutes	50.00 (7.14)**	54.16 (7.42)**	27.08 (31.33)*	35.41 (36.50)*
SL_3	Mercuric chloride 0.1% for 2 minutes followed by ethyl alcohol 70% for 10 seconds	54.16 (7.42)**	52.08 (7.28)**	83.33 (65.87)*	81.24 (64.33)*
SL_4	Mercuric chloride 0.1% for 5 minutes followed by ethyl alcohol 70% for 10 seconds	81.24 (9.06)**	85.41 (9.29)**	56.24 (48.57)*	66.25 (54.48)*
SL_5	Carbendezim 0.02% for 20 minutes + S_3	56.24 (7.56)**	56.24 (7.56)**	87.50 (69.26)*	89.58 (71.19)*
SL_6	Carbendezim 0.02% for 30 minutes + S_3	79.16 (8.95)**	77.08 (8.83)**	60.41 (50.99)*	75.00 (59.97)*
SL_7	Carbendezim 0.02% for 20 minutes + S_4	95.83 (9.84)**	91.66 (9.62)**	50.00 (44.98)*	60.41 (50.99)*
SL_8	Carbendezim 0.02% for 30 minutes + S_4	97.91 (9.94)**	95.83 (9.84)**	50.00 (44.98)*	56.24 (48.57)*
	$C.D_{(P\leq0.05)}$ Cultivar (C)	NS		0.67	
	Sterilant (S)	0.16		1.34	
	CX S	0.23		1.89	

*Figures in the parentheses are the statistically arcsin transformed values of percentage data
**Figures in the parentheses are the statistically square root transformed values of percentage data

Conclusion

An effective sterilization of bulb scale and leaf segment explants of LA Hybrid cultivars of Lilium, "Indian Summerset" and "Nashville" was carried out in the present investigation wherein, significant results of culture asepsis and survival percentage were obtained when Carbendazim, Mercuric chloride and Ethanol were used in combination at different concentrations and duration of exposure. In both the cultivars, highest asepsis was recorded in SB_8 treatment combination in case of bulb scale explant and for leaf segment explant SL_8 treatment combination gave highest asepsis per cent. Maximum survival of bulb scale explants was observed in SL_7 treatment combination and for leaf segment explants highest survival was recorded in SL_5. Similar findings were observed in both the cultivars.

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